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PHARMACOLOGICAL SCREENING AND STANDARDIZATION OF NEW POLYHERBAL FORMULATIONAS AN ANTIDIABETIC PHYTOTHERAPEUTICS

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ABSTRACT

Objective: Emerging medicinal plants like *Annona squamosa*, *Morus alba* and *Nelumbo nucifera*are individually used in treatment of diabetes mellitus. The Antidiabetic Activity of these emerging medicinal plants is well known as an individual antidiabetic plant but the effect of their combination is still unclear. Here the study is designed to develop the polyherbal formulation of leaves extract of only this emerging Antidiabeticmedicinal plants with fruits extract of piper longum as bioavailablity enhancer and screened for antidiabetic potential in Streptozotocin (60 mg/kg) induced diabetic rats. The investigating formulation was also evaluated for quality control parameters i.eMarker based High Performance Thin Layer Chromatography (HPTLC), Heavy metal content determination, Microbial load. The quality control parameters were evaluated as per the WHO guidelines of herbal drugs.

Methods: Two dose level 200 and 400 mg/kg of formulation was selected for Oral glucose tolerance test and antidiabetic activity against the standard drug Glibenclamide (5mg/kg). Acute toxicity study of formulation did not showed any toxic symptoms up to the dose level of 2000mg.kg body weight. Formulation was administered daily for 15 consecutive days and blood glucose level was measured at 0 hr., after 3 hr., 5th day, 10th day and 15th day. At the end of study the blood samples were collected and tested for biochemical parameters Total Cholesterol, Total Triglycerides, High density lipoprotein, Low density lipoprotein (TC,TG,HDL-C,LDL-C), Urea Creatinine. Blood glucose level was determined by glucometer (Escencia entrust, Bayer Health Care).

Results: Study showed that new polyherbal formulation with a dose of 400mg/kg was found extremely significant (65.8%) in reducing the blood glucose level and this was also supported by the result of biochemical analysis of blood samples. HPLTLC analysis showed the effective separation at 366 nm and also revealed the presence of active component in the formulation. Total microbial load, heavy metal content was found to be within the limits of WHO guidelines.

Interpretation and Conclusions: Synergistic effect of this polyherbal combination showed the very fruitful result in reducing the blood glucose level i.e 65.8% from 0 to 15 days which is very near to the Standard drug Glibenclamide which showed 66.2% decrease in blood glucose level from 0 to 15 days.

KEYWORDS: *Annona squamosa*, *Morus alba* and *Nelumbo nucifera*, Polyherbal, Antidiabetic Activity, HPTLC Standardization, Quality Control Parameters

INTRODUCTION

Herbals are very useful to mankind. The demand of herbal medicines are increasing day by day. In developing countries 80% of population still believe on traditional herbal medicines for treatment of diseases. Our ancient literatures has described about 500-800 medicinal plants which have been used in aboriginal system of medicines. According to the World Health Organization (WHO) "a medicinal plant is a plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." Medicinal plants are depot of active ingredients which can be used for number of purposes in traditional healing system of medicine (Huai 2010; Husain et al 2008). The various indigenous system of medicine such as Siddha, Ayurveda, Unani and Allopathy has been using the several medicinal plant species to treat many ailments; diabetes mellitus is also one of them. Diabetes mellitus is most common disorders now days which are affecting the millions of people worldwide. According to International Diabetes Federation's (IDF) estimates, 80% of the world diabetic population will be from low- and middle-income countries in 2030 (Petchi et al 2013). Treatment protocol of diabetes mellitus includes different category of drugs such as sulfonylurea, biguanides, thiazolidinediones, alpha-glucosidase inhibitors etc. but major problem are adverse effect with these drugs in treating the systemic disorder. Hence many research institutes and pharmaceutical companies are inclined towards the herbal medicines and investigating the drug molecule having desired therapeutic potential with less adverse effect (Parasuraman et al 2010). A large number of medicinal plants used in the treatment of diabetes and their individual antidiabetic effect are well known but their synchronous effect in polyherbal is unpredictable. In Ayurveda the concept of polyherbalism has been mentioned, it provides the better synergistic therapeutic effect and less adverse effect. Here the study was designed to develop and evaluate the polyherbal of these emerging antidiabetic plants in animal models and establish their quality control parameters.

MATERIALS AND METHODS

Collection of Plant Materials

Leaves of *Annona squamosa*, *Morus alba*, *Nelumbo nucifera*, *Psidium guajava* and fruits of *Piper longum* were collected from village of Barabanki district Uttar Pradesh India. The collected plants were authentified at National Botanical Research Institute (NBRI) Lucknow. The voucher specimens of the plants were deposited at Department of Pharmacology NBRI for further reference.

Animals

25 Albino wistar rats (150-200g) of either sex were obtained from Indian Institute of Toxicological Research (IITR) Lucknow for antidiabetic study. Animals were housed in polypropylene cages at an ambient temperature of 25-30 °C and 45-55 % relative humidity with a 12 hr each of dark and light cycle in an animal house of King George Medical College Lucknow. Animals were fed pellet diet and water *ad libitum*. The study was approved by Institutional Animal ethical committee of King George Medical College Lucknow and all the experiments were carried out as per the committee approval for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Ministry of Environment and Forests, Government of India.

Preparation of Extracts and Design of Polyherbal Formulation

The plant materials were washed thoroughly with distilled water, dried, grinded, powered and extracted with ethanol: water (40:60) in soxhlet apparatus. The resulted hydro alcoholic extract was filtered and concentrated in a rota

vapour under reduced pressure to give a semisolid residue. Polyherbal Formulation was developed by incorporating equal dose (100 mg) of different extracts of emerging antidiabetic medicinal plants as shown in table 1.

Acute Oral Toxicity Study

Acute oral toxicity study of polyherbal formulation were carried out as per the guidelines of Organization for Economic Co-operation and Development (OECD) no.423.As per OECD guidelines minimum number of animals should be used (3 animals per dose) for experiment to obtained the information on Acute toxicity of test dose. Overnight fasted rats were orally fed with polyherbal formulation at a dose level of 250, 500, 1000 and 2000 mg/kg body weight, respectively. The animals were observed continuously for 2hr to investigate any sign of toxicity, occasionally for 4 hr for their general behavior and after a period of 24 hr, animals were observed for any sign of mortality till 14 days. Hence two dose levels were selected for antidiabetic study i. e 200 mg ($1/10^{th}$ of maximum toxic dose) and 400 mg (twice of I^{st} dose).

Antidiabetic Activity

Diabetes was induced in rats by intraperitonial injection of Streptozotocin (60 mg/kg b.wt) dissolved in normal saline. Diabetes was confirmed by measuring the blood glucose level by glucometer (Escencia entrust, Bayer health care) after 72 hrs. of Streptozotocin administration. Blood samples were drawn by picking the rat tail. The diabetic rats with blood glucose levels ≥250 mg/dl were selected for the study. After 72 hr of STZ injection animal exhibiting Blood glucose level ≥ 250 mg/dl were divided in to 5 groups (with 5 animals each) for antidiabetic study of Formulations (Alimohammadi Samad et al 2013; BaragobAbdellawt al 2014)

Following groups were prepared:

Group I: Normal control given distilled water

Group II: Negative control (treated with STZ 60 mg/kg b.wt i.p)

Group III: Standard (Treated with Glibenclamide 5mg/kg b.wt after 3rd day of STZ injection)

Group IV: Treated with Formulation orally dose of 200 mg/kg b.wt after 3^{rd} day of STZ injection)

Group V: Treated with Formulation orally dose of 400 mg/kg b.wt after 3^{rd} day of STZ injection)

From 3^{rd} day, Standard drug and Formulation was given daily for 15 days and blood glucose was measured on 3^{rd} day (assume as 0 day), 5 th day, 10^{th} day and 15^{th} day of study with the help of Glucometer (Ascensia ENTRUST, Bayer Health Care). Blood sample was taken by picking the rat tail vein for the measurement of Blood glucose level (BGL).

Biochemical Parameters

At the end of 15^{th} day blood sample was withdrawn from retro-orbital plexus of rats for the measurement of biochemical parameters.2-4 ml of blood was collected and centrifuged at the 5000 rpm for 15-20 min and serum was taken out with the help of syringe and was analyzed for other biochemical parameters (Total cholesterol, Total triglycerides, HDL, LDL, SGOT, SGPT, Urea, Creatinine) through auto analyzer.

Assessment of Quality Control Parameters

Polyherbal formulation consists of number of ingredients which causes batch to batch variations Hence

establishment of quality control parameters is a basic requirement for the safe use of herbal formulation.

HPTLC Profiling

DESAGA Sarstedt Gruppe system is used for analysis along with Automatic TLC applicator and UV visible cabinet as imaging system, the instrument had ProQuant 1.6 version as software system for documentation. Hydro alcoholic extract of polyherbal formulation was taken on aluminum plate coated with silica gel 60 F254 of 0.2 mm thickness (E. Merck) as adsorbent. Experimental conditions was temperature 25 ± 2 ºC, and relative humidity 40 % The mobile phase used was Toluene: Ethyl acetate (17.1:3.5 v/v).The plate was dried and visualized under UV 254 nm and 366 nm. The spraying agent used was Anisaldehyde with 5 % H_2SO_4.

Preparation of Sample Solutions and Standard Solution of Gallic Acid

A stock solution of standard compound and sample was prepared by dissolving 0.1 mg of accurately weighed standard (Gallic acid, Piperine and Stigmasterol) and sample in 5ml of methanol.

Development of TLC Plate

Exactly 10µl of standard and sample were spotting on the TLC plates with the help of TLC applicator. The Plates were developed in solvent system of Toluene: Ethyl acetate (17.1:3.5 v/v) in TLC chamber previously saturated with solvent system for 30 min. After that the plates were dried in air and scanned with Densitometer CD60 of DESAGA Sarstedt Gruppe system under the UV range of 366 nm, 254 nm, and visible region. The Densitogram obtained as shown in figure 1 and 2 with peak areas and spots number of corresponding the concentration of sample.

Safety Profile

Heavy Metal Content Determination by Atomic Absorption Spectroscopy

Polyherbal formulation was tested for presence of heavy metal content by acid digestion method (Bushra, etal 2011; Sugnya etal 2012). Estimation of heavy metals (Lead, Arsenic, Cadmium and Mercury) was done by Atomic absorption spectrometry in Biotech Park Lucknow, results were found within the permissible limits of WHO guidelines.

Microbial Screening

As one of safety parameter of herbal drug total aerobic microbial count of polyherbal formulation was determined by Plate count method as per Ayurvedic Pharmacopeia of India Vol II 2.4.1 at Biotech Park Lucknow.

For Bacteria Count

Soya bean casein digest media was used for total bacteria count.10 g of sample was diluted with buffered sodium chloride-peptone solution pH 7.0 and volume was make up to 100 ml. 2 petri dish were used. In each petri dish, mixture of 1ml of sample was added with 15 ml of liquefied casein soya bean digest agar at 45°C. Sample was spread properly on the surface solidified medium in petridish and was incubated it at 30°C to 35°C for 5 days. After 5 days the numbers of colonies were counted (taking 300 colonies per plate as the maximum consistent).

For Fungi Count

Same procedure was followed with the use of Sabouraud Dextrose Agar with Antibiotic (Chloramphenicol) and petri dish were incubated at 20° to 25°C for 5 days..(Taking 100 colonies per plate as the maximum consistent)

RESULTS AND DISCUSSIONS

Acute Oral Toxicity

Toxicity study did not showed any mortality sign up to the dose of 2000 mg.kg body weight Hence the study was carried out at 200 mg/kg body weight and 400 mg/kg body weight.

Antidiabetic Screening

Glucose levels measured in blood of normal and experimental rats are given in Table 2. Diabetic control rats showed significantly increased in blood glucose level (BGL). Polyherbal formulation showed a significant reduction in the blood glucose level of diabetic rats on daily oral administration with a slight decrease in body weight. The mean blood glucose level in diabetic control on 0 day was 307.4±7.31mg/dl and on 15^{th} day was 359.4±5.99 mg/dl. It was observed that the standard drug glibenclamide showed significant reduction in blood glucose level and resuscitatesit to normal level. It was observed that standard drug showed average decrease in BGL (66.2%) from 0 day to 15^{th} day with maximum decrease to 58.6 mg/dl between 5^{th} to 10^{th} day whereas the polyherbal formulation at 200 mg/kgand 400 mg/kg showed significantly decreased the fasting blood serum glucose level i. e 62.9% and 65.8% in the diabetic rats from 0^{th} day to 15^{th} day as shown in table 3. It was observed that throughout the study period of 15 days, dose 200 mg/kg showed maximum reduction (37.26%) in average BGL between 10^{th} to 15^{th}day whereas dose 400 mg/kg showed maximum reduction (43.8%) between 10^{th} to 15^{th} day. At the end of study it was noticed that maximum reduction in Blood glucose level to 65.80 % was seen at 15 day with a dose of 400 mg/kg which was very much near (i.e 66.2%) to standard drug glibenclamide 5m/kg. At the end of study serum of animals was analyzed for biochemical parameters and it was observed that serum TG, Total cholesterol, LDL-cholesterol were found to be increased significantly ($P<0.001$) in STZ induced diabetic rats as compared to non-diabetic control as shown in table 4. HDL cholesterol was found to be significantly decreased in diabetic rats. Treatment with formulation produces a significant reduction in elevated serum TG, TC, LDL-cholesterol level in diabetic rats. Maximum Increase in HDL level was found to be 57.12% (greater than standard) with a dose of 400 mg/kg b.wt of polyherbal formulation (table 5).

Quality Control Parameters

HPTLC Profiling

Fingerprinting analysis showed effective separation at 366nm and 254 nm (figure 6). The obtained R_f values and peak areas in densitometry TLC scanner are shown in (table 6-10 and figure 1-5).Under 254 nm it showed 4 spots with R_f value at 0.04, 0.22, 0.27, 0.50 in which R_f value 0.04 and 0.50 corresponds to standard Gallic acid and Stigmasterol; under 366 nm it showed 5 spots with R_f values at 0.11, 0.15, 0.24, 0.42 and 0.48.R_f value 0.15 corresponds to the standard compound Piperine.

Heavy Metal Determination by AAS

It was observed that heavy metal lead and arsenic present in polyherbal formulation was found to be within the limit as prescribed in Ayurvedic Pharmacopeia of India and cadmium and mercury was not detected as shown in table 11.

Microbial Load Analysis

For the safe use of polyherbal formulation microbial testing was done and microbial count was found to be within the limits (table 12) as per API 2.4.1.

CONCLUSIONS

Thus our study demonstrates that polyherbal formulation producean effective antidiabetic effect at 400mg/kg which is very near to allopathic drug glibenclamide. Hence more research is required on standardization parameters for this polyherbal combination so that it can be proved as an effective and safe antidiabetic phytotherapeutics.

REFERENCES

1. Alimohammadi Samad**,** Hobbenaghi Rahim**,** Javanbakht Javad**,** Kheradmand Danial**,** Mortezaee Reza**,** Tavakoli Maryam**,** Khadivar Farshid and Akbari Hamid (2013). Protective and antidiabetic effects of extract from *Nigella sativa* on blood glucose concentrations against Streptozotocin (STZ)-induced diabetic in rats: an experimental study with histopathological evaluation. Diagnostic Pathol. 8, 137
2. Baragob Abdella Emam Abdella, Malki Waleed Al Hassan, and EldeenIzz Omar Muhammad (2014). The hypoglycemic effect of the aqueous extract of the fruits of *Balanitesaegypticea* in Alloxan-induced diabetic rats Pharmacognosy Research. 6(1), 1-5.
3. Gupta K Rajesh, Kesari N Achyut, WatalGeeta, Murthy P.S, Chansra Ramesh, MaithaliKapli, Tondon Vibha.(2005) Hypoglycemic and antidiabetic effect of aqueous extract of leaves of *Annona squamosa* (L.) in experimental animal. Curr. Sci.88, 25.
4. Habeeb N, NajmaNiak, Prakash R., FahmiS Moqbel (2012) Inhibition of alpha glucosidase and alpha amylase by *Morus alba* Linn leaf extracts. J Pharm Res.5, 285.
5. Hina Bushra, Rizwani Ghazala Hafeez and NaseemShahid. (2011) Determination of toxic metals in some herbal drugs through Atomic Absorption Spectroscopy. Pak. J. Pharm. Sci. 24,353-358.
6. Huai H. (2010) Ethno medicinal analysis of toxic plants from five ethnic groups in China. Ethnobot Res Appl. 8,169–79.
7. Husain SZ, Malik RN, Javaid M, Bibi S. (2008) Ethonobotanical properties and uses of Medicinal plants of Morgah Biodiversity Park, Rawalpindi. Pak J Bot. 40, 1897–911.
8. Huralikuppi C J, Christopher B A, Stephen M P (1991) Anti-diabetic effect of *Nelumbo nucifera* (Gaertn): Part I preliminary studies in rabbits. Phyto. Res. 5,54–58.
9. Kang Joo Myung, Cho Youl Jae, Shim Ho Byung, Kim Ki Duk and Jaehwi Lee (2009). Bioavailability enhancing activities of natural compounds from medicinal plants. Med Plant Res.3, 1204-1211.
10. Parasuraman S, Kumar E, Kumar A, Emerson S. (2010) Free radical scavenging property and diuretic effect of triglize, a polyherbal formulation in experimental models. J Pharmacol Pharmacother. 1, 38–41.
11. Patil K Umesh, Singh Amrit, Chakraborty K Anup. (2011)Role of Piperine as a Bioavailability Enhancer. Inter. J. Recent advan Pharm. Res.4, 16-23.

12. Petchi RR, Parasuraman S, Vijaya C.(2013) Antidiabetic and antihyperlipidemic effects of an ethanolic extract of the whole plant of *Tridaxprocumbens* (Linn.) in Streptozotocin-induced diabetic rats. J Basic Clin Pharm.4, 88–92.
13. Sudha P**,** Zinjarde S Smita**,** Bhargava Y Shobha, Kumar R Ameeta. (2011)Potent α-amylase inhibitory activity of Indian Ayurvedic medicinal plants BMC Comp Alter Med.11, 5.
14. Suganya P, Radha R, Jayshree N, Selvakumar S.(2012)Evaluation of Antiurolithiatic Polyherbal Formulation (Capsule). J Pharmacog Herb. Formul. 2, 20-27.

APPENDICES

Table 1: Design of Polyherbal Formulation

Plant Added	Part Used	Reported Mode of Action for Antidiabetic Activity	Quantity (mg)
Annona squamosa	Leaves	Antioxidant, increase in insulin secretion(Gupta et al,2005)	100
Morus alba	Leaves	α-glucosidase and alpha amylase inhibitor(**Habeeb et al, 2012;Sudha et al,2011)**	100
Nelumbo nucifera	Leaves	Increase glucose utilization(Huralikuppi et al,1991)	100
Psidium guajava	Leaves	alpha amylase inhibitor (**Sudha et al,2011)**	100
Piper longum	Fruits	Bioavailablity enhancer(Patil et al,2011; Myung Joo Kang et al,2009**)**	100

The data were expressed as mean ± SEM. The data were analyzed by two way ANOVAs

Table 2: Effect of Formulation on Blood Glucose Level in STZ Induced Diabetic Rats (Long Term Study of 15 Days Daily Once)

CHANGE IN FASTING BLOOD GLUCOSE LEVEL						
Groups	**Fasting Blood Glucose Level (mg/dl) After 18 hrs.**	**After 3rd Day of STZ Injection (0 hr/0 day) mg/dl**	**After 3 hr (mg/dl)**	**Day 5th (mg/dl)**	**Day 10th (mg/dl)**	**Day 15th (mg/dl)**
Normal Control	69.6±3.94	77.6±2.99	77.4±2.15	76.4±1.07	78.0±1.14	80.2±1.35
Diabetic Control	68.2±2.15	307.4±7.31	308±7.31	327.2±7.84	343.6±6.20	359.4±5.99
Standard Glibenclamide (5mg/kg)	66.8±2.22	294.6±16.87	266.8±15.33	212.4±13.51	153.8±11.59	99.4±5.37
Test F (200mg/kg)	70.6±1.63	327±15.92	307±16.27	249±12,62	193.2±8.98	121.2±6.89
Test F (400mg/kg)	69.8±1.42	304±11.62	280±11.54	221±10.03	163.8±6.77	103.8±3.62
P values < 0.001 Values are Mean ± SE from 5 animals in each group.						

Table 3: Total Decrease in Average % of Blood Glucose Level from 0 Day to 15 Day

Treatment	Dose (mg/kg b.wt)	Average Decrease in BGL from 0 Day to 15th Day	Remarks
Standard(Glibenclamide)	5 mg/kg	66.2%	Showed maximum decrease in average BGL to 58.6 mg/dl between 5th to 10th day
Polyherbal Formulation	200 mg/kg	62.9%	Showed maximum decrease in average BGL to 72 mg/dl (37.26%) between 10th to 15th day
	400 mg/kg	65.8%	Showed maximum reduction in average BGL to 60 mg/dl(43.8%)between 10th to 15th day

Table 4: Effect of Formulation on Biochemical Parameters of Blood. Values are Mean ± SE from 5 Animals in each Group

Parameters	Normal Control		Diabetic Control		Standard Glibenclamide		Test F 200 mg		Test F 400 mg	
	0th Day	After 15thDay	0th Day	After 15thDay	0th Day	After 15th Day	0th Day	After 15thDay	0th Day	After 15th Day
Cholesterol	52.7 ±2.65	52.66 ±2.25	95.24 ±4.79	112.78 ±6.20	107.08 ±6.49	48.3 ±3.14	150.18 ±7.11	82.42 ±6.06	157.36 ±9.51	83.84 ±7.34
Triglycerides	66.68 ±3.03	68.1 ± 3.51	112.6 ±14.7	146.62 ±17.53	133.9 ±10.81	66.42 ±4.32	137.68 ±12.20	71.06 ±4.73	145.72 ±5.90	75.84 ±4.71
SGOT	20.36 ±3.77	19.54 ±3.88	59.88 ±4.15	70.28 ±4.13	54.54 ±3.96	19.26 ±2.34	76.08 ±5.04	32.96 ±2.71	84.68 ±3.40	37.56 ±4.91
SGPT	23.5 ±2.91	26.26 ±2.64	63.6 ±2.35	77.34 ±3.12	70.42 ±1.00	20.76 ±1.32	76.20 ±3.55	39.16 ±2.47	72.94 ±3.02	29.30 ±2.60
Creatinine (mg/dl)	0.63 ±0.12	0.71 ± 0.11	1.55 ±0.03	1.78 ±0.04	1.71 ±0.07	0.93 ±0.10	1.63 ±0.07	0.84 ±0.08	1.61 ±0.08	0.68 ±0.08
Urea(mg/dl)	26.9 ±1.48	27.48 ±1.40	74.54 ±9.17	82.08 ±7.57	80.0 ±4.15	27.1 ±2.83	142.32 ±10.64	108.74 ±11.11	128.60 ±12.51	99.16 ±13.15
HDL (mg/dl)	37.32 ±1.34	38.34 ±1.24	22.08 ±2.06	13.5 ±1.41	20.82 ±2.46	38.18 ±1.12	19.50 ±2.53	35.94 ±3.12	15.22 ±1.97	35.50 ±1.86
LDL (mg/dl)	25.72 ±1.57	27.32 ±2.17	67.2 ±3.95	72.6 ±3.55	62.16 ±2.45	25.00 ±1.42	73.82 ±3.04	52.68 ±3.14	75.72 ±3.23	63.28 ±3.99

P values: <0.001 significantly different from Control, Diabetic control

Table 5: Average Decreases in Biochemical Parameters from 0 to 15 Days

Treatment	Dose	Cholesterol	Triglycerides (TG)	SGOT	SGPT	Creatinine	Urea	HDL	LDL
Glibenclamide	5mg/kg	54.8%	50.3%	64.6%	70.5%	45.6%	66.1%	45.4%	59.7%
F-D	200mg/kg	41.1%	37.96%	56.67%	48.60%	48.16%	22.89%	45.7%	28.70%
	400mg/kg	46.7%	48.38%	55.60%	59.83%	57.46%	23.59%	57.12%	16.42%

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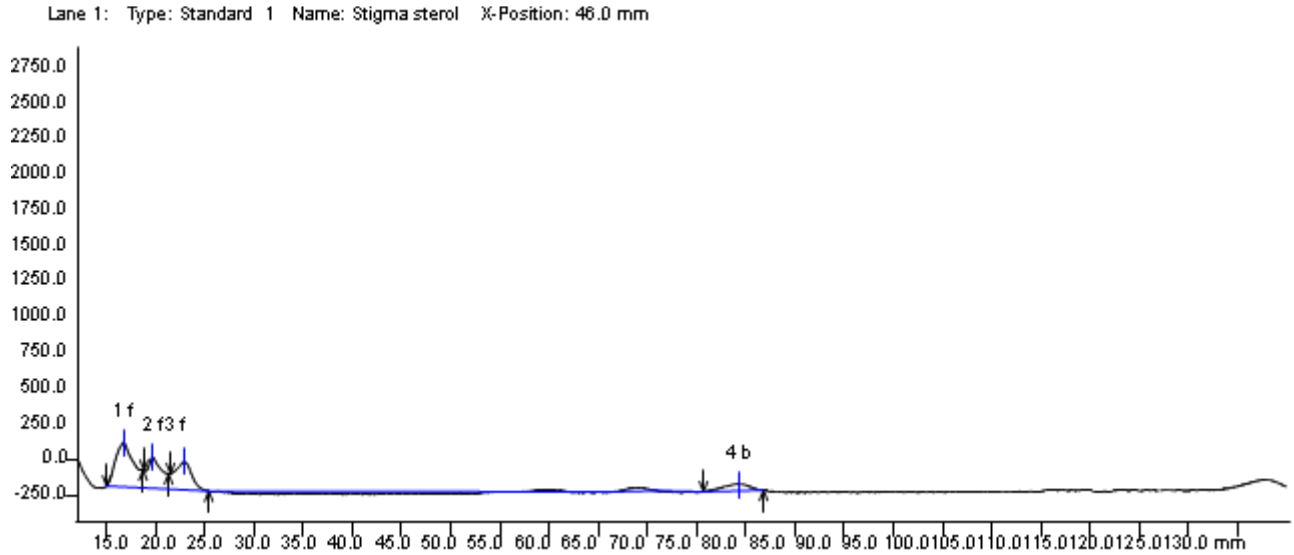

Figure 1: Chromatogram of Standard Compound Stigmasterol at 254 nm

Table 6: Chromatogram Analysis (Peak Area and Peak Height) of Standard Stigmasterol at 254 nm

Lane: 1 Type: Standard 1 Name: Stigma Sterol X-Position: 46.0 mm							
Peak	**Component Name**	**y-Pos [mm]**	**Area**	**Area[%]**	**Height**	**Type**	**Rf**
1	**:**	**16.7**	**656.57**	**42.4**	**308.30**	**f**	**0.01**
2	**:**	**19.7**	**363.36**	**23.5**	**217.25**	**f**	**0.03**
3	**:**	**22.9**	**378.84**	**24.5**	**194.08**	**f**	**0.06**
4	**: Stigma sterol**	**79.3**	**148.64**	**9.6**	**46.65**	**b**	**0.53**

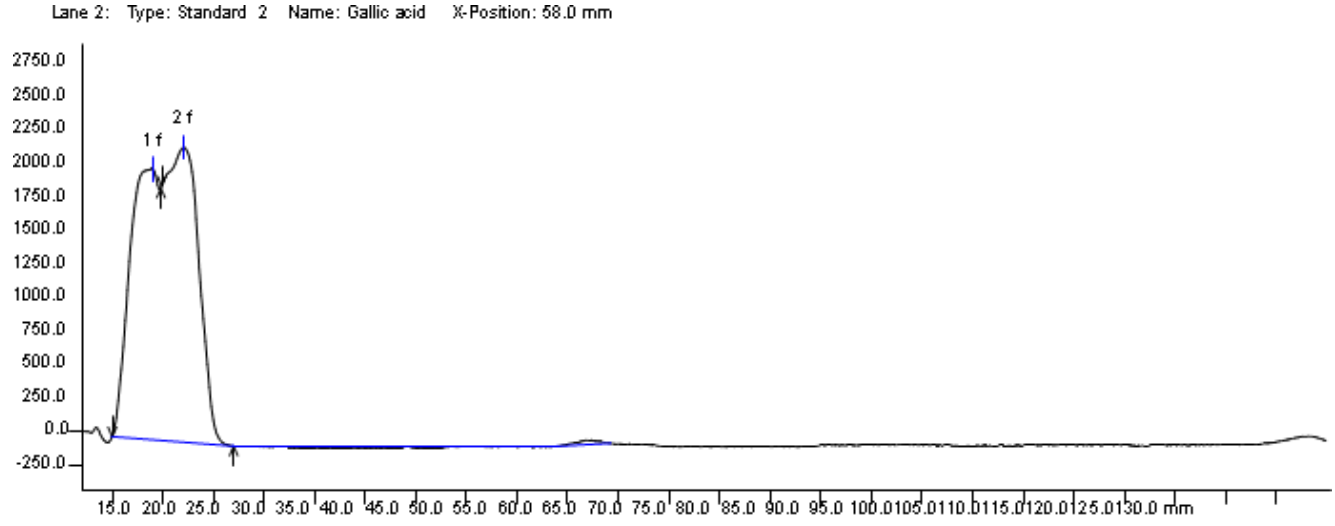

Figure 2: Chromatogram of Standard Compound Gallic Acidat 254 nm

Table 7: Chromatogram Analysis (Peak Area and Peak Height) of Standard Gallic Acid at 254 nm

Lane: 2 Type: Standard 2 Name: Gallic Acid X-Position: 58.0 mm							
Peak	**Component Name**	**y-Pos [mm]**	**Area**	**Area[%]**	**Height**	**Type**	**Rf**
1	**:**	**19.0**	**6629.54**	**44.2**	**2015.32**	**f**	**0.03**
2	**: Gallic acid**	**22.1**	**8354.15**	**55.8**	**2194.08**	**f**	**0.05**

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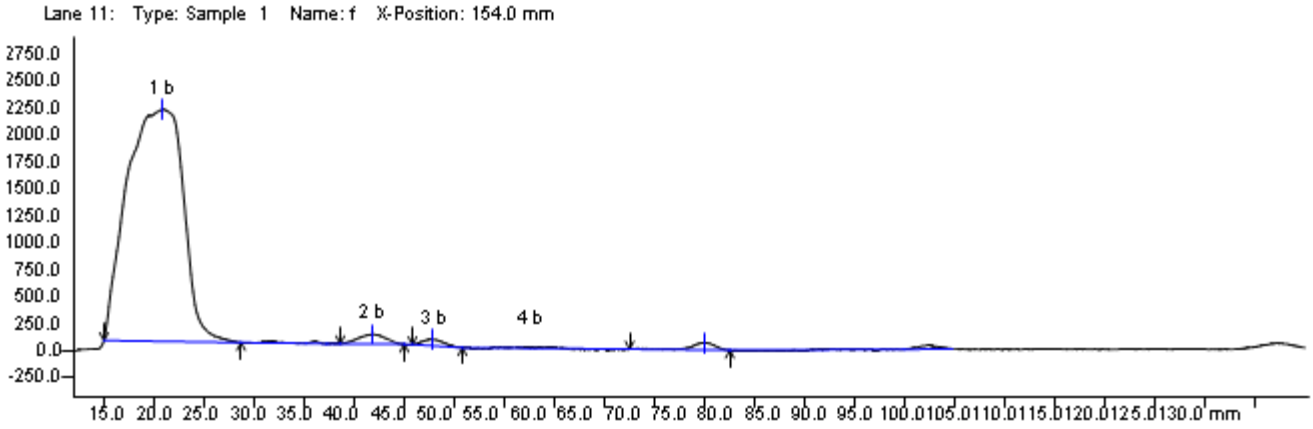

Figure 3: Chromatogram of Polyherbal Formulationat 254 nm

Table 8: Chromatogram Analysis (Peak Area and Peak Height) of Polyherbal Formulation at 254 nm

Lane: 11 Type: Sample 1 Name: Formulation X-Position: 154.0 mm							
Peak	**Component Name**	**y-Pos[mm]**	**Area**	**Area[%]**	**Height**	**Type**	**Rf**
1	**: Gallic acid**	20.9	1400.19	95.8	2145.74	b	0.04
2	**:**	41.8	281.93	1.9	89.26	b	0.22
3	**:**	47.9	144.88	1.0	58.37	b	0.27
4	**: Stigma sterol**	75.0	18.64	1.3	67.59	b	0.50

DESAGA ProQuant: Densitogram + Peaklist			
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Created by: USER		**Date/Time:**	28-Nov-13 04:32:26 PM
Comment:			
Method: Method for Chromatogram		**ID-Number:**	04798-1385674346-8
Created by: USER		**Date/Time:**	8-Nov-13 03:26:02 PM

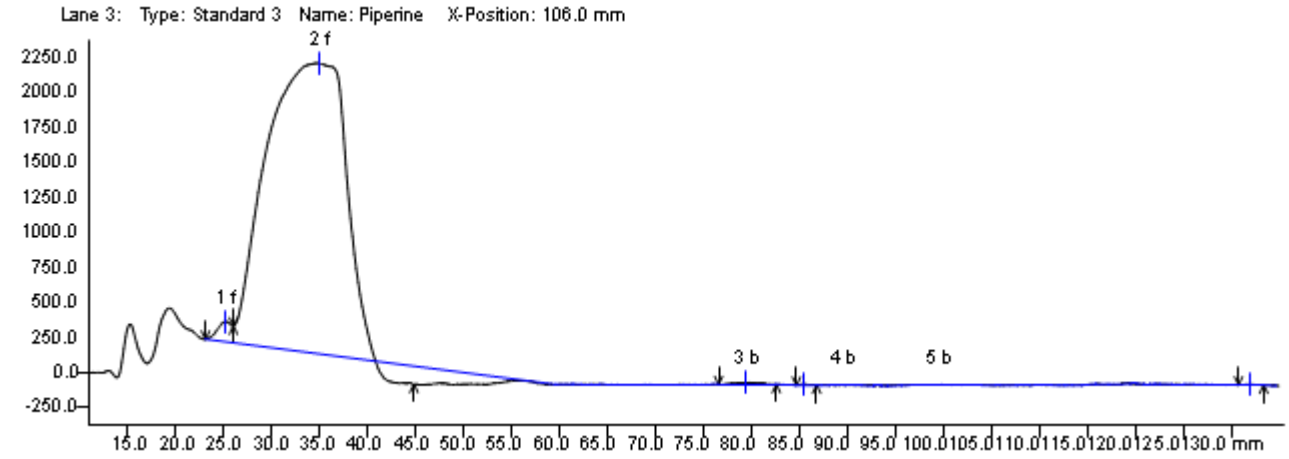


Figure 4: Chromatogram of Standard Compound Piperineat 366 nm

Table 9: Chromatogram Analysis (Peak Area and Peak Height) of Standard Piperine at 366 nm

Lane: 3 Type: Standard 3 Name: Piperine X-Position: 106.0 mm							
Peak	**Component Name**	**y-Pos [mm]**	**Area**	**Area[%]**	**Height**	**Type**	**Rf**
1	**:**	**25.3**	**239.72**	**1.2**	**144.99**	**f**	**0.08**
2	**:**	**35.1**	**19774.60**	**98.5**	**2073.45**	**f**	**0.16**
3	**:**	**79.5**	**43.13**	**0.2**	**14.82**	**b**	**0.53**
4	**:**	**85.4**	**6.33**	**0.0**	**7.58**	**b**	**0.58**
5	**:**	**131.9**	**6.83**	**0.0**	**6.45**	**b**	**0.97**

DESAGA ProQuant: Densitogram + Peaklist	
Chromatogram: Measurement - Method for Chromatogram	**ID-Number:** 04798-1385674346-8
Created by:	**Date/Time:** 28-Nov-13 04:32:26 PM
Comment:	
Method: Method for Chromatogram	**ID-Number:** 04798-1385674346-8
Created by:USER	**Date/Time:** 28-Nov-13 03:26:02 PM

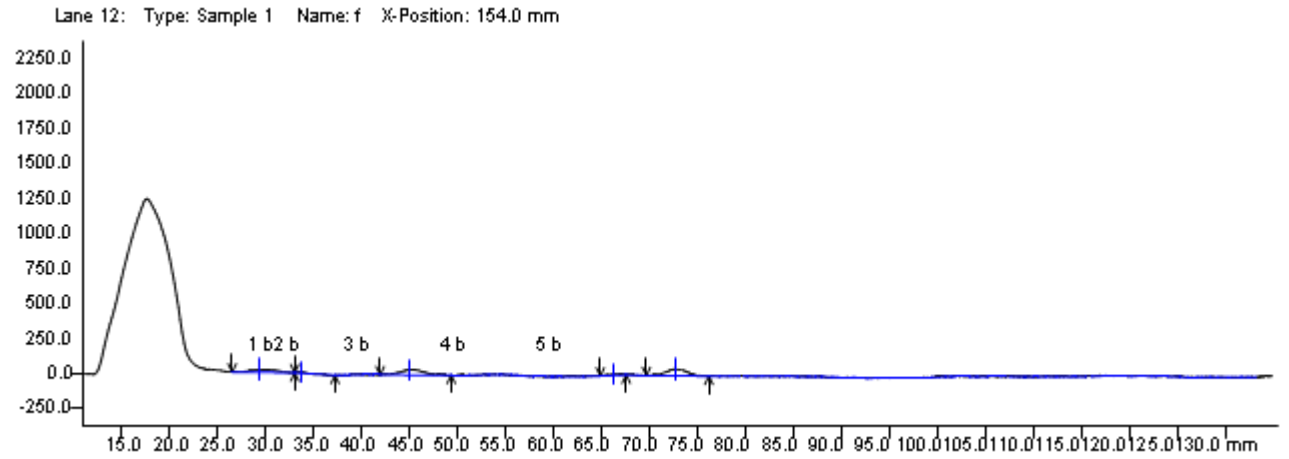


Figure 5: Chromatogram of Polyherbal Formulationat 366 nm

Table 10: Chromatogram Analysis (Peak Area and Peak Height) of Polyherbal Formulation at 366 nm

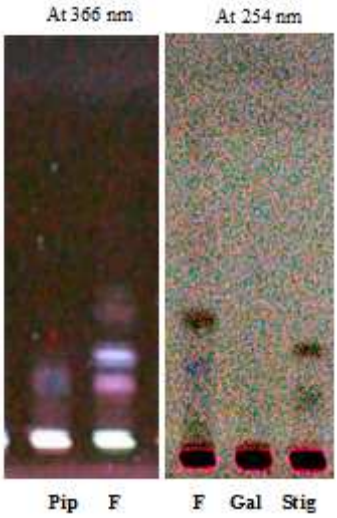
Lane: 12 Type: Sample 1 Name: Formulation X-Position: 154.0 mm							
Peak	**Component Name**	**y-Pos [mm]**	**Area**	**Area[%]**	**Height**	**Type**	**Rf**
1	:	29.5	64.21	20.3	20.51	b	0.11
2	: **Piperine**	33.8	7.15	2.3	9.71	b	0.15
3	:	45.0	109.98	34.7	37.40	b	0.24
4	:	66.2	12.26	3.9	7.18	b	0.42
5	:	72.8	123.22	38.9	46.75	b	0.48

Table 11: Heavy Metal Analysis of Formulation

Sample	Lead (Pb) µg/ml	Cadmium (Cd)µg/ml	Mercury (Hg) µg/ml	Arsenic (Ar) µg/ml
Polyherbal	2.8	Not Detected	Not Detected	0.7

Table 12: Total Aerobic Microbial Count

Parameter Tested	Method	Result
Total bacterial count	A.P.I 2.4.2	Less than 10^1 CFU/ml
Total fungal count		2×10^1CFU/ml



F = Formulation, Pip = Piperine, Gal = Gallic Acid, Stig = Stigmasterol

Figure 6: TLC of Polyherbal Formulation at 366nm and 254 nm